

The University of Chicago

THE FORMATION OF THYROXINE
FROM ARTIFICIALLY IODIZED
PROTEINS

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
AUGUST, 1940

By
LEMUEL CALVERT CURLIN

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INTRODUCTION

After the discovery of iodine in thyroid tissue by Baumann (1) in 1895, many attempts have been made to prepare iodized proteins with thyroid activity. Uniformly negative physiological results were reported by Hoffmeister (2) on iodized egg white, by Blum and Vaubel (3) on iodized casein, and by Oswald (4) on several iodized proteins. Blum (5) reported positive and negative results with iodized albumin. Diiodotyrosine, according to Morse (6), and certain fractions from hydrolyzed iodized proteins, according to Mattis (7) and Brandt (8), hastened metamorphosis in tadpoles.

Schwaibold (9) claimed that feeding iodized protein to frog larvae had no effect on metamorphosis, but that feeding the products of pepsin and trypsin digestion of the iodized proteins acted as a strong thyroid preparation.

Abelin (10), in a series of papers, showed that the method and extent of iodizing proteins is of particular importance in determining whether the protein is highly reactive or entirely unreactive. Upon hydrolysis, Abelin's active iodized proteins yielded an active, acid-insoluble and an inactive, acid-soluble fraction. Although he was unable to isolate crystalline thyroxine, he showed that his active fractions possessed the biological and chemical properties of crude thyroxine. He called his product "homo-thyroxine", and was able to isolate it from iodized casein, egg albumin, serum albumin and globulin, and peptone. He claimed, however, that peptones and ereptones gave a much lower yield.

In 1936, Ludwig and von Mützenbecher (11) obtained a patent on the production of crystalline thyroxine from iodized protein and in a later paper (12) they outlined their procedure. Their isolation of thyroxine from iodo-casein was confirmed by Harington (13).

This was the answer to the nature of the thyroid-like activity of iodized proteins, but the mechanism of thyroxine formation was not clear. Harington (14) had earlier suggested that thyroxine is formed in the thyroid gland by coupling of two diiodotyrosine molecules and this explanation was favored by Ludwig and von Mützenbecher (12). But, as Harington stated (13), "There seems an inherent unlikelihood that iodine should bring about such a drastic oxidation and a greater improbability that a reaction of the type required should occur between two tyrosine residues within the protein molecule."

As an alternative to this mechanism there was the possibility of thyronine existing, preformed, in the protein molecule and its iodization to thyroxine. Abderhalden (15) had observed that feeding thyronine and potassium iodide to guinea pigs was more effective in raising the metabolic rate than feeding either alone.

In an attempt to answer these questions on the mechanisms of thyroxine formation, two lines of experimentation were undertaken: first, the search for thyronine from protein hydrolysates; second, the iodization of tyrosine and tyrosine derivatives to thyroxine.

PART I

Procedure:

Our preliminary studies and the results of Harington (16) show that thyronine and tyrosine have similar chemical and physical properties. Attempts to separate the two amino acids by fractional crystallization of their hydrochlorides, their mercury salts, and the free amino acids, yielded negative results. Although thyronine and its derivatives showed different solubilities to those of tyrosine when in the pure form, this difference did not appear to be sufficient to enable us to separate these two amino acids from the mixtures representative of those calculated to be present in the protein on the assumption that thyroid activity was due to a preformed thyronine fraction.

In an attempt to form the alpha hydroxy compound of the amino acids, it was noted that thyronine gave an orange color instantly with nitrous acid, whereas tyrosine developed this color only after 15 minutes. These colored compounds were separated by extraction with ether and their ultraviolet adsorption spectra were taken, but no characteristic absorption spectra were noticed.

Absorption curves of thyronine and tyrosine solutions of 4×10^{-6} molar, were run for us by Professor Hogness of the Department of Chemistry. The maximum absorption for tyrosine was 2879 and that for thyronine was 2950 Angstrom units. These peaks were, therefore, too close for use in detecting thyronine in the presence of tyrosine and other amino acids.

Beta naphthoquinone-4-sulfonate reacts with amino acids to form a deep brown colored solution. Attempts to separate these colored derivatives of tyrosine and thyronine by chromatographic adsorption failed.

The separation (17) of cholesterol and ergosterol by chromatographic adsorption of their azoyl esters (azobenzene carboxylic acid), suggested a method for the separation of thyronine from tyrosine. The ethyl esters of the amino acids, were used for coupling with azoyl chloride, because they are more soluble in organic solvents and also, because much better yields were obtained than with the free amino acid.

The adsorption method was varied with the amount of material to be adsorbed. For small samples, simple filtration through a 1 x 20 cm. glass tube, with one end constricted and plugged with glass wool and filled with adsorbing material, was used. For larger amounts of material, a pressure apparatus was used (18).

After adsorption, the separated substances were removed from the adsorbant by continuous extraction with chloroform in a Soxhlet extractor. The chloroform solution was evaporated to a small volume and the substance precipitated with 60-68 degree Skelly-solve.

Experimental:

Preparation of Azoyl Esters:

Azoyl chloride was prepared by the methods of Ladenburg, Fernholz and Wallis (17) and of Reich (19). The latter procedure gave better yields, but the method is longer and greater care must be taken to have anhydrous reagents.

Ethyl esters of the amino acids were prepared by repeatedly treating the compounds with absolute ethanol and with dry

hydrogen chloride gas.

The azoyl esters of tyrosine, thyronine, and tyrosine fractions from protein hydrolysates were prepared similarly. 1.5 gms. of dl-tyrosine ethyl ester hydrochloride were dissolved in 5 cc. of water, 15 cc. of chloroform were added and the mixture was cooled to -5° in an ice-salt bath. The free ester was liberated by adding slightly more than the equivalent amount of 5 N sodium hydroxide (1.5 cc.), with mechanical stirring. 1.5 gms. of azoyl chloride were dissolved in 10 cc. of chloroform and added in portions over a period of 2 minutes. Then, 3.6 cc. of 2 N sodium carbonate were added and again 1.5 gms. of azoyl chloride, dissolved in 10 cc. of chloroform, were added in portions. The mixture was stirred for 5 minutes longer, then transferred to a separatory funnel. The chloroform layer was washed twice with dilute sodium carbonate solution and once with water and dried over anhydrous sodium sulfate. The chloroform was evaporated in vacuo until crystals began to form. Then 3-4 volumes of 60-68° Skelly-solve were added. After standing for half an hour the precipitate was centrifuged down and washed three times with Skelly-solve to remove unreacted azoyl chloride. This process yields about 85% of the theoretical. The loss is due to the hydrolysis of the tyrosine ethyl ester before coupling with the azoyl group. Attempts to avoid this hydrolysis were undertaken, by coupling the azoyl chloride and the tyrosine or thyronine esters in pyridine at 0° , 10° , room temperature, and at the boiling point of pyridine. Additions of azoyl chloride to a pyridine solution of the ester and of the ester to a pyridine solution of azoyl chloride, were run. The compounds were precipitated by pouring into cold water and neutralizing with hydrochloric acid or by distilling off the pyridine under reduced pressure and recrystallizing

the compounds from ethyl acetate. No increase of yield over the aqueous method was found and many by-products were formed due to the action of pyridine on the amino acids.

Solvents for Developing Solution and Choice of Adsorbants:

Azoyl tyrosine and thyronine ethyl esters are insoluble in petroleum ether; slightly soluble in benzene, carbon tetrachloride, ether, and alcohol; quite soluble in both chloroform and acetone. Mixtures of chloroform and 60-68° petroleum ether (Skelly-solve) were chosen for developing the chromatogram.

Preliminary studies with three adsorbents; bentonite, magnesium oxide, and aluminum oxide, prepared by heating Bauxite ore concentrate Grade 33, led to the latter as the best choice. This was obtained from the Aluminum Corporation of America. Washing this heated product with methyl alcohol seemed to increase its adsorbing power, but most of the runs were made with the unwashed preparation.

Separation of the Pure Azoyl Compounds by Chromatographic Adsorption:

Azoyl tyrosine ethyl ester and azoyl thyronine ethyl ester were prepared as described in 85-90% yields. Azoyl tyrosine ethyl ester-m.p.-178°, Nitrogen-- 9.9%, theory-- 10.1%. Azoyl thyronine ethyl ester-m.p.-142°, Nitrogen-- 8.0%, - Theory -- 8.2%.

Two mg. of the thyronine compound and 200 mg. of the tyrosine compound were dissolved in a minimum amount of chloroform. The column was prepared by suspending the dry powder (200 mesh) in a mixture of 3:1 chloroform: Skelly-solve and pouring into the glass tube. By tapping the sides of the glass tube and putting pressure on the top of the column, the powder was firmly packed and all air bubbles were removed. Care was taken to keep

some liquid above the top of the powder. Better rings could be obtained if a pinch of glass wool was packed on the top of the column. This prevented the disturbance of the surface of the powder while refilling the tube with liquid. The solution of the two compounds was poured on the column and immediately two rings began to form. Fifty cc. of 3:1 chloroform Skelly-solve mixture was poured through and the lower ring moved down the column, while the upper ring remained motionless. The lower ring was washed into a receiver, then the column was cut and the upper ring was removed and the compound extracted in a Soxhlet with chloroform. The chloroform was evaporated from both solutions until crystals began to form and then 3-4 volumes of petroleum ether (Skelly-solve) were added and the precipitated compounds centrifuged and dried in vacuo. Upper ring compound--m.p.--178°, Yield-----150 mg. Lower ring compound--m.p.--142°, Yield-----1.8 mg.

Separation of Tyrosine and Thyronine through the Azoyl Compounds:

Fifty mg. of thyronine were mixed with 1 gm. of tyrosine and the ethyl ester hydrochlorides of the mixture were prepared as described earlier. Yield--100%. The ester hydrochlorides were coupled with azoyl chloride and the mixture of azoyl compounds separated as described. Yield--95%. The chromatographic adsorption and fractionation gave two rings which yielded: Upper ring (Azoyl tyrosine ethyl ester) m.p.---178°, Yield---0.72 gm. Lower ring (a mixture) m.p.---145-225°, Yield---0.4 gm.

The lower ring compound was dissolved in chloroform and poured through another column prepared as before. One ring formed. 3:1 mixture of chloroform and Skelly-solve was poured through the column and a small upper ring and larger lower ring formed. The lower ring was washed through with the developing

solvent and the upper ring was washed through with chloroform. Upper ring (Azoyl thyronine ethyl ester) m.p.----145^o, Yield----10 mg. Lower ring m.p.----235^o, Yield----200 mg. This lower ring compound on analysis proved to be diazoyl tyrosine ethyl ester.

The compounds were very difficult to remove from the aluminum oxide after adsorption. This accounts for the low recoveries.

Recovery of Thyronine Added to Casein:

Five mg. of thyronine were added to 50 gm. of commercial casein and the mixture was stirred with 320 cc. of water. One hundred sixty gm. of barium hydroxide (8 H₂O) and 0.5 cc. of caprylic alcohol were added and the mixture was refluxed for 24 hours. The hot solution was filtered and the excess barium hydroxide crystallized out by cooling in the refrigerator. The crystals were filtered off and recrystallized from hot water; the filtrates were combined and heated to boiling. Sodium sulfate was added in excess and after digesting on the steam bath for an hour, the barium sulfate was filtered off and washed with hot water. The filtrates were combined and evaporated to 250 cc. The solution was neutralized and made slightly acid to litmus with acetic acid and was let stand in the refrigerator for two days. The precipitate was filtered off and redissolved in 2% sulfuric acid. After filtration, the mixture was adjusted to pH 5.5 and crystallized in the ice-box. Yield--1.9 gm. This precipitate was dried for two hours at 110^oC., esterified with absolute ethanol and dry hydrogen chloride as described above and finally converted into the azoyl compound, using twice the equivalent quantity of azoyl chloride. Yield--3.5 gm.

An aluminum oxide column was prepared and chromatographic

adsorption and separation led to these fractions: Upper ring (azoyl tyrosine ethyl ester) m.p.---178°, Yield----2.5 gm. Lower ring A (diazoyl tyrosine ethyl ester) m.p.---235°, Yield----0.4 gm. Lower ring B (azoyl thyronine ethyl ester) m.p.---145°, Yield----1.0 mg.

This experiment was repeated, yielding essentially the same results.

Attempts to Isolate Thyronine from Casein as the Azoyl Compound:

A. One hundred gm. of commercial casein were hydrolysed by the method described above and the tyrosine fraction isolated. Yield---3.8 gm. This was dried, esterified, and converted into the azoyl compound. Yield---6.3 gm. On chromatographing it yielded two fractions. Upper ring (azoyl tyrosine ethyl ester) m.p.---178°, yield----3.0 gm. Lower ring (diazoyl tyrosine ethyl ester) m.p.---235°, yield----0.4 gm.

The lower ring was run through another column, but only one ring was formed even after washing with 3:1 chloroform, Skelly-solve mixture. The recovered compound gave a melting point of 235°.

B. One thousand gm. of commercial casein were run through the same procedure. Thirty-eight gm. of tyrosine were isolated. This was esterified and the azoyl compound was made of 15 gm. of the ester hydrochloride. Yield---31 gm. This compound was chromatographed on a large column, using pressure above the chamber to hasten the filtration. Two rings formed, which were extracted and the resulting compounds were precipitated and dried. Upper ring (azoyl tyrosine ethyl ester) m.p.---178°, yield----21 gm.; lower ring (diazoyl tyrosine ethyl ester) m.p.---235°, yield----3 gm.

The lower ring was run through another column, but only

one ring separated.

Reduction and Hydrolysis of Thyroglobulin:

Brandt and Kassel (20) hydrolyzed thyroglobulin with sodium hydroxide and sodium stannite and claim that this process converts thyroxine into a compound which gives a Millon test. They assume the compound to be either diiodothyronine or thyronine. It was hoped that our method would enable us to isolate thyronine from the mixture and prove the usefulness of our method in isolating thyronine from natural sources.

Fifty gm. of dried thyroglobulin were refluxed with 1000 cc. of 5% stannous chloride in 5.5 N sodium hydroxide for 30 hours, with a slow passage of nitrogen through the mixture. Caprylic alcohol was added to prevent foaming. The solution was filtered while hot and then 500 cc. of 5 N sodium hydroxide and 38 gm. of zinc dust were added. After stirring and warming to 50°, 3 more portions of zinc dust were added and the finely divided precipitate was filtered off. The solution was diluted to 3 liters with water and neutralized with acetic acid. The solution was warmed to 70° and 95 gm. of mercuric acetate and 40 cc. of glacial acetic acid were added. After digesting on the steam bath for 3 hours, 300 gm. of sodium chloride were added and the mixture placed in the refrigerator over night. The supernatant fluid was decanted and the precipitate centrifuged and washed with 200 cc. of water. Five hundred cc. of 2 N sulfuric acid were added to the precipitate and this was digested for 1 hour, and while still warm, hydrogen sulfide was added under pressure. The mixture was shaken for 5 hours and the black mercuric sulfide was filtered off. The filtrate was made slightly acid to litmus and let stand over night in the ice-box. A gray precipitate was filtered off and dried. Yield---1.2 gm. This product was

esterified and the azoyl compounds were made. An adsorption column was set up and a chloroform solution of the compounds was poured on the column. A lower pink band and a larger upper band was formed. Nothing could be recovered from the lower pink band and the upper band was the tyrosine compound.

Discussion:

Thyronine, added to casein in amounts equivalent to thyroxine isolated from iodized casein, can be separated as the azoyl ethyl ester. However, no thyronine could be detected in pure casein.

PART II

Procedure:

As was stated earlier there are two possibilities whereby thyroxine might be synthesized during the iodization of casein. In Part I, above, we have shown that thyronine, preformed, could not be isolated from casein, by a method which would detect added thyronine. Therefore, tyrosine is iodized and changed in some way to thyroxine. Since earlier synthesis of diiodotyrosine had not produced thyroxine as a by product, it was necessary to see if tyrosine could be changed to thyroxine without being coupled to a protein molecule.

Experimental:

Confirmation Thyroxine Formation in Iodo-casein:

The isolation of thyroxine from iodo-casein according to Ludwig and von Mützenbecher (12) was confirmed and 100 mg. of thyroxine were obtained from 100 gm. of iodo-casein. The thyroxine from several preparations was catalytically reduced to thyronine by the method of Ludwig and von Mützenbecher.

Iodization of Casein Hydrolysate:

To see whether tyrosine needed to be linked to the protein molecule in casein for thyroxine formation, casein was hydrolysed and the hydrolysate iodized after the manner of Ludwig and von Mützenbecher. Their procedure for the isolation of thyroxine was followed, but no thyroxine was isolated. Large amounts of tar, or melanin, were formed during the iodization of the hydrolysate, which might have prevented the separation of

thyroxine:

Iodization of Tyrosine:

Abelin (10) had shown that the yield of "homo-thyroxine" from iodized proteins depended to some extent on the degree of iodization. Ludwig and von Mützenbecher had found that thyroxine was obtained in better yields when the protein was not saturated with iodine and that complete iodization led to much tar formation. After repeated attempts to isolate thyroxine from tyrosine, iodized with equivalent quantities of iodine, we made runs with less iodine. Still a large amount of tar was formed. Application of the Leland and Foster (21) butyl alcohol method to the tar, in our hands, led to the separation of thyroxine from fully iodized and incompletely iodized dl-tyrosine.

Ten gm. of dl-tyrosine, obtained from casein, were warmed to 38°C. in 3 liters of water, 100 gm. of sodium bicarbonate were added, and 20 gm. of iodine (theory--28 gm.) were slowly added while stirring mechanically over a period of four hours. After all the iodine was added, the mixture was stirred for two hours longer and allowed to stand over night at 38°. Then the mixture was acidified with sulfuric acid to pH 5, the precipitate allowed to settle and filtered off. The brown amorphous precipitate was taken up in 200 cc. of 4 N sodium hydroxide, which contained 5% sodium carbonate, and this solution was extracted with 200 cc. of butyl alcohol. The butyl alcohol layer was extracted with 100 cc. of 4 N sodium hydroxide and then the alcohol was distilled off under reduced pressure. The residue was taken up in water and precipitated with hydrochloric acid at pH 4.5. After centrifuging the precipitate, it was dried in vacuo. Yield 0.25 gm. This brown substance was dissolved in the least amount of 0.1 N sodium carbonate by heating, and sodium thyroxinate crystallized out on

cooling to 0° . The crystals were centrifuged down and washed with a little cold sodium carbonate solution. The crystals were taken up in 75% alkaline alcohol, filtered free of tar, heated to boiling and precipitated with 33% acetic acid. Yield 1 mg. m.p. $--231-233^{\circ}$ (dec.)

Seventy gm. of dl-tyrosine, from casein, were iodized and treated according to the above procedure. 1.5 gm. of a brown powder were obtained by the butyl alcohol procedure and 5 mg. of thyroxine were isolated.

A few months after these experiments, a paper by von Mützenbecher (22) appeared, describing the preparation of thyroxine by incubation of diiodotyrosine at pH 8.6. This work we confirmed as follows:

Diiodotyrosine was prepared by dissolving dl-tyrosine in 7.5% ammonium hydroxide and adding an equivalent amount of N iodine. The solution was made acid to congo red and filtered free of a brown tar. The filtrate was brought to pH 5.5 with sodium hydroxide and allowed to crystallize in the ice-box. The brown crystals were filtered off and dried. Yield--75% of theory.

Twenty gm. of the dl-tyrosine were mixed with 420 cc. of water and brought to pH 8.8 with sodium hydroxide. The solution was placed at 38° for two weeks. At this time, the pH had dropped to 8.05. The solution was diluted to 4 liters and made weakly acid with dilute sulfuric acid. A light brown amorphous precipitate formed, which settled in two hours. The supernatant liquid was decanted and the precipitate was centrifuged. This was dissolved in 100 cc. of 2 N sodium hydroxide and extracted twice with 100 cc. of butyl alcohol, the butyl alcohol extractions combined, and evaporated to dryness under reduced pressure. The residue was taken up in 50 cc. of water and acidified with acetic

acid. After standing over night, the yellow precipitate was centrifuged and taken up in the least amount of hot 0.1 N potassium carbonate solution, filtered, and cooled to 0°. The fine white precipitate was centrifuged down and dissolved in 80% alkaline alcohol, heated to boiling, and precipitated by adding 33% acetic acid. The white amorphous precipitate was centrifuged down and dried. Yield--20 mg., m.p.---235° (dec.) Iodine--64.8%, theory---65.4; Nitrogen---1.82, theory---1.80.

This procedure was also used for the preparation of thyroxine from tyrosine directly. 8.35 gm. of tyrosine were mixed with 420 cc. of water and buffered at pH 9.7 with sodium hydroxide and sodium bicarbonate. The temperature was raised to 38° and 23 gm. of finely powdered iodine were added, in portions, to the mechanically stirred solution during a period of two hours, and stirred for two hours longer. The pH was now 8.65. The solution was incubated for two weeks at 38° and at the end of this time the pH had dropped to 8.25. After dilution to 4 liters, the solution was made weakly acid with sulfuric acid and a dark green precipitate settled out. The supernatant fluid was decanted and the residue centrifuged down and taken up in 100 cc. of 2 N sodium hydroxide and extracted with butyl alcohol. Thyroxine was isolated as described above. Yield---22 mg., m.p.---235° (dec.)

Attempts at Increasing the Yield of Thyroxine:

von Mützenbecher (22) states that the addition of sodium sulfite to the incubating diiodotyrosine solution, inhibits the formation of both thyroxine and the amorphous substance which we have called tar. This inhibition by sulfite seems to point to an oxidative process and attempts were made to increase the yield of thyroxine by adding oxidizing agents.

To 20 gm. of diiodotyrosine, incubated as described

earlier, 0.5 gm. of powdered iodine was added daily for 10 days. Thyroxine was isolated as described before, in approximately the same yield. About twice the amount of amorphous material was formed as before.

Preparation of l-Thyroxine:

l-Tyrosine was prepared by the enzymatic hydrolysis of casein and l-diiodotyrosine was prepared from this, by the ammoniacal method, as mentioned before. Twenty gm. of l-diiodotyrosine were incubated as before and 25 mg. of thyroxine were isolated. This product was recrystallized 3 times by dissolving in 80% alkaline alcohol and precipitating with 33% acetic acid. Yield--17 mg. This was dissolved in 2 cc. of a mixture of 6 cc. of 0.5 N sodium hydroxide and 14 cc. of 95% ethanol. A 1 cc. micro-polaroscope tube was used. $[\alpha]_D^{20} = -3.5$. Considering large experimental errors in measuring such a small rotation, this value checks well with Harington's (23) value of -3.0.

Pancreatic Digestion of Diiodotyrosine:

Salter (24,25,26), in a series of papers, has described the enzymatic synthesis of an artificial protein, which relieves clinical myxedema, from diiodotyrosine peptones. Although Harington (13) has shown that enzymes are not involved in the activation of iodo-casein, we hoped that addition of enzymes to the incubating diiodotyrosine would cause an increased yield of thyroxine.

Twenty gm. of diiodotyrosine were mixed with 800 cc. of water and brought to a pH of 8.2 with sodium hydroxide. The solution was placed in an incubator at 38° and 0.5 gm. of pancreatin was added daily for 10 days. The pH on the fourth day was 8.0, on the tenth day it was 7.2. At this time, the solution was heated to the boiling point and filtered while hot to free from

protein. After cooling, the solution was made weakly acid with dilute sulfuric acid and the dark precipitate settled out in two hours. The supernatant fluid was decanted and the precipitate was centrifuged and dissolved in 100 cc. of 2 N sodium hydroxide. The butyl alcohol extraction and separation of thyroxine was carried out as described before. Yield---35 mg.

During the first precipitation of thyroxine as potassium thyroxinate, fine silky crystals were noticed in the supernatant fluid. These crystals are being studied in our laboratory and, at present, seem to be a thyroxine poly-peptide.

Thyroxine from Synthetic Tyrosine:

Since the yield of thyroxine from diiodotyrosine is very low, there was still a possibility of thyroxine being formed from traces of thyronine in the dl-tyrosine obtained from casein. To disprove this theory, dl-tyrosine was synthesized (27), converted into diiodotyrosine and after digestion with mild alkali as before, thyroxine was isolated. Five gm. of synthetic diiodotyrosine gave 4.5 mg. of thyroxine. m.p.--233-235° (dec.)

Discussion:

From the yields obtained, it is obvious that per molecule of diiodotyrosine involved we formed approximately 6 times as much thyroxine when iodo-casein was used, as compared with diiodotyrosine. Also, the addition of pancreatin to the digest, seemed to increase the yield of thyroxine from diiodotyrosine. In fact, the incubation of diiodotyrosine with pancreatin not only led to a higher yield of thyroxine, but to the formation of what appears to be a thyroxine poly-peptide.

Thyroxine was formed from synthetic tyrosine, which makes improbable the possibility that thyroxine is formed from thyronine impurities in natural tyrosine.

SUMMARY

1. Thyronine was added to casein in amounts equivalent to the amount of thyroxine separated from iodo-casein. By hydrolysis, azoyl ester formation, and chromatographic separation, thyronine was separated as the azoyl thyronine ethyl ester. No thyronine, however, could be separated from casein alone.

2. The ultraviolet absorption curves of tyrosine and thyronine are described.

3. The orange color produced by nitrous acid, developed much more rapidly with thyronine than with tyrosine.

4. The formation of thyroxine, from iodized casein, is confirmed.

5. The preparation of thyroxine, by iodizing dl-tyrosine, is described.

6. The formation of thyroxine, by incubation of diiodo-tyrosine with alkali at 38° , is confirmed.

7. The preparation of thyroxine from synthetic dl-tyrosine is described.

8. Since thyronine could not be isolated from proteins, by a method which permitted the separation of added thyronine from casein, it is believed that the mechanism of thyroxine formation from iodized proteins involves two molecules of diiodo-tyrosine.

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